THE IMMUNOLOGICAL REACTIONS OF THE PROTEINS OF THE HUMAN PLACENTA WITH SPECIAL REFERENCE TO THE PRODUCTION OF A THERAPEUTIC SERUM FOR MALIGNANT CHORION-EPITHELIOMA.*

GLEASON C. LAKE.

(From the Pathological Laboratory of the University of Chicago.)

Various authors have reported the production of immune sera, which they believed to be specific for certain cells or cell complexes. The work to be reported in this article was undertaken to determine whether antisera specific for human placenta, or for certain of its proteins, could be obtained. The placenta, especially the fetal portion, is known to behave in many respects as a tissue foreign to the mother, and therefore seemed to offer a more favorable field for such work than the permanent and essential tissues of the body. It was hoped that a specific antiserum to placental tissue, if it could be produced, would be of particular value in the treatment of chorion-epithelioma, either alone or as an adjunct to surgical procedure. Considerable work has already been reported concerning the production of specific cytolytic antisera by the injection of nucleoproteins of organs, which in some cases have been stated to have a therapeutic value. Beebe has been the chief exponent of this work, which, however, has not been confirmed by the work of Pearce and others.[1] In view of these facts, this work, then, seemed desirable (1) as a repetition of the investigation of immune reactions of the nucleoproteins (which can hardly be considered as definitely settled); (2) because little work has been done with the proteins of the placenta, the nucleoprotein being the only one so far used; and (3) because the special phase of this problem, the application of antiplacental sera to the therapy of chorion-epithelioma, had not been considered.

The literature on the subject is not very extensive. So far as the placenta itself is concerned, we have an excellent review by

* Received for publication February 2, 1914.

[1] See review by Wells, *Ztschr. f. Immunitätsf.*, 1913, 19, p. 599.

Frank[1] in 1907, covering the subject to that time. I can do no better than give a brief summary of Frank's paper.

Halban had accumulated numerous clinical observations, from which he adduced that the growth of the placenta causes a hypertrophy and hyperemia of the uterus and neighboring organs. The only experimental work in support of this was that of Lane-Claypon and Starling, who found that rabbit placenta injected into virgin rabbits produces hypertrophy of the breasts. Kollman had observed that the fetal syncytium underwent lysis in the maternal fluids. Scholten and Veit tried to prove this experimentally by immunizing rabbits with human placenta; they reported producing an antiserum which dissolved placental cells *in vitro*. Leipman was unable to confirm this work. He found, however, that such an antiserum contained precipitins for placenta and placental blood, but admitted that the washed placenta contained general body proteins as well as special placental cell proteins, and that absorption of the "general" proteins was first necessary to differentiate them from the specific placental proteins. Weichardt, Opitz, and Wormser, in attempting to repeat Leipman's work, obtained negative results; while Kawasoye and Freund report results similar to those of Leipman. Frank's work, which appears thorough and adequately controlled, showed that injections of rabbit placenta into rabbits produced no antibodies, as shown by the precipitin test. Injection of human placental "nucleoprotein," with a content of 0.35 per cent phosphorus, prepared as nearly as possible blood-free, did not produce any antibodies that could be detected by the precipitin and complement fixation tests. Immunization with suspensions of blood-free placental cells, however, did produce antisera as shown by the precipitin, complement fixation, agglutination, and hemolytic tests, but at no time did the antisera show any demonstrable cytolytic action. None of these reactions, however, was specific for placental cells, and they are referred to by Frank as weak "human reactions." In his conclusions he states that "nucleoproteins act merely as mild toxic agents, without specific qualities."

Since the appearance of this work in 1907 several articles have been published which have a bearing on the subject, especially as regards the individuality of the fetus in relation to the mother. Zuntz[2] has shown this by the specific character of the enzymes of the fetus and placenta. He mentions the work of other authors showing that there is a difference in the agglutinative power of maternal and fetal serum. The work of Abderhalden on specific enzymes for placental proteins is too well known to need more than mention.

Anderson and Rosenau[3] have reported the sensitization of guinea-pigs to guinea-pig placental extract, with pronounced anaphylactic symptoms resulting when the intoxicating dose of the same material was given, indicating that the placenta is biologically foreign to animals of the same species. They suggested that in some cases eclampsia might be explained on this basis. Thies[4] and Gräfenburg,[5] after an extensive

[1] *Jour. Exper. Med.*, 1907, 9, p. 263.
[2] *Ergeb. d. Physiol.*, 1908, 7, p. 403.
[3] *Jour. Med. Research*, 1908, 14, p. 37.
[4] *Arch. f. Gynäk.*, 1910, 92, p. 513.
[5] *Ztschr. f. Geburtsh. u. Gynäk.*, 1911, 69, p. 270.

series of experiments, held that during pregnancy the mother is sensitized by small amounts of fetal protein and that if a considerable quantity of fetal blood is at one time introduced into the mother anaphylactic symptoms follow. Johnstone[1] and Fellander[2] were unable to confirm these results and Williams, Lynch, and other obstetricians do not believe that this is the true explanation of eclampsia.

These experiments, while not in harmony, at least suggest the biochemical individuality of the fetus and placenta, the bearing of which is at once evident on our problem, as it would indicate that if the placental material could be freed from other tissues and blood, specific antisera might be produced of value in the treatment of eclampsia and of malignant chorion-epithelioma. Risel[3] and others have shown that spontaneous healing of the chorion-epithelioma occurs in a certain percentage of cases. The fact that this healing tendency is never seen to an equal degree with other malignant tumors is highly suggestive that placental tumor cells act as a foreign substance, to which the host may react with considerable success by means of antibody formation. Removal of the primary tumor is also said to be followed often by the healing of distant metastases. Hence the malignant placental tumors would seem to offer a better prospect for treatment by antisera than other forms of malignant tumors, which arise from essential tissue cells and which do not show so marked a tendency to spontaneous retrogression or healing.

Stimulus to work along the line of the production of specific cytotoxic sera in recent years is perhaps attributable more to the work of Beebe than to any other investigator. He used other tissues than placenta, but the general principles involved are the same. Beebe[4] found that animals injected with the "nucleoproteins" of various organs yielded an antiserum of high specificity, e.g., rabbits immunized to dog liver nucleoprotein produced an antiserum reacting specifically with this protein, as shown by the precipitin and agglutination tests. Injection of this serum into dogs produced characteristic lesions in the liver. Proteins from the thymus, kidney, pancreas, and other organs were also used.

[1] *Jour. Obst. and Gynec. Brit. Emp.*, 1911, 19, p. 253.

[2] *Zischr. f. Geburtsh. u. Gynäk.*, 1911, 68, p. 26.

[3] *Ergeb. d. Allg. Path. u. Path. Anat.*, 1907, 11, Part 2, p. 928.

[4] *Jour. Exper. Med.*, 1905, 7, p. 733.

It should be mentioned that in a later communication[1] he says that it is not a simple matter to produce immune serum to nucleoproteins; e.g., of 5 rabbits only 1, and of 4 sheep only 1 produced active sera. As to specificity, he states that the serum has two factors, a general and a specific, and that to show the specific lesions in an animal receiving these sera, it must be killed at just the right time. Beebe[2] later extended his work and produced an immune serum for thyroid nucleoprotein, and used it in the treatment of goiter, obtaining results of some promise. Rodgers[3] reported a series of 10 cases treated in this manner, three of which were greatly benefited or cured. Taylor[4] made an antiserum for thyroid nucleoprotein according to Beebe's methods, but it did not give therapeutic results of value.

Bierry[5] reports that rabbits injected with nucleoprotein of dogs' kidney develop an antiserum, nephrotoxic to dogs. He states, however, that normal serum injected in the same amount produces some of these symptoms, tho not so marked. He does not state the effect of immunizing rabbits to dog serum and then trying this antiserum as a control.

Fiessinger[6] reported the production of an antiserum to liver and kidney nucleoprotein which showed some degree of relative specificity. Not all the work, however, gives such positive results. Pearce and Jackson,[7] working with the nucleoproteins prepared in a different manner (containing 1.74–2.1 per cent phosphorus), were unable to confirm Beebe's work. Beebe[8] criticized these authors in that in the preparation of their material it was brought to the boiling point, altho it has often been shown that proteins which are not coagulated and rendered insoluble are usually not altered in their antigenic properties by the amount of heating which these nucleoproteins received. Beebe again emphasizes the specificity of his products, stating that the precipitin and also the agglutination reactions were still obtained, tho in less degree, after the absorption of the general antibody content of the serum by treating it with dog muscle. He does not give complete protocols showing the dilutions in which these reactions occurred, nor whether they were always controlled with simultaneous tests on dog serum; nor does he mention treating dogs with serum of rabbits immunized to dog serum. He states that "the most searching and conclusive method, however, is by means of animal inoculations." It would seem, on the contrary, that if the active principle of the serum is an antibody, specificity could be more easily and certainly demonstrated by the precipitin and complement fixation reactions. The work of Nuttal, von Behring, Uhlenhuth, and others would lead us to that conclusion. Wells,[9] working with nucleoproteins prepared from dog liver by Beebe's method, found only a considerable toxicity, and no specific anaphylaxis reaction could be obtained. When

[1] *Brit. Med. Jour.*, 1906, 2, p. 1786.
[2] *Jour. Am. Med. Assn.*, 1906, 46, p. 484.
[3] *Ibid.*, 1906, 46, p. 487.
[4] *Ibid.*, 1911, 56, p. 263.
[5] *Compt. rend. Soc. de biol.*, 1903, 55, p. 476.
[6] *Jour. de physiol. exper.*, 1908, 10, p. 657.
[7] *Jour. Infect. Dis.*, 1906, 3, p. 742.
[8] *Jour. Am. Med. Assn.*, 1910, 55, p. 1212.
[9] *Jour. Infect. Dis.*, 1911, 9, p. 147.

guinea-pigs were given an intoxicating dose of dog serum after being previously sensitized with nucleoprotein, a moderate reaction was obtained. This held only when relatively large sensitizing doses of the nucleoprotein had been given. No reaction was obtained when only 1–2 mg. of nucleoprotein were given in the sensitizing dose. Finally, the most complete, as well as the most recent work, is that of Pearce, Karsner, and Eisenbrey.[1] Their materials, while not prepared exactly according to the method of Beebe, were open to none of the objections of the earlier work of Pearce and Jackson. The immune sera were tested by the precipitin, hemolytic, and agglutination reactions, none of which gave evidence of any specificity of the sera. The fractions, as tested by the anaphylactic method, showed a very slight relative organ specificity, but none for the different protein fractions of the same organ. Microscopic study of the organs of dogs receiving the various antisera gave no support to the view of specific action. They conclude that nucleoproteins play no important rôle in the production of cytolytic immune sera.

Doerr[2] gives an excellent review of the literature as regards specificity in anaphylaxis. He states that specificity of organs is difficult to demonstrate, as it is difficult to get organs free from serum. In case of tissues easily freed from serum, as the crystalline lens of the eye, the nails, etc., proteins may be prepared which show highly specific properties. These are the exceptions. The work of Wells and Osborne[3] shows that the question of specificity depends on the chemical composition of the proteins; that species specificity simply means that the material is really of demonstrably different chemical composition, and that where the composition is identical by the most delicate chemical methods, differing species specificity is not exhibited; and, on the other hand, that several chemically different proteins from the same source, animal or vegetable, may act as entirely distinct specific antigens. This is very well shown by the work with hen egg proteins.[4] Here Wells was able to demonstrate the presence in the hen's egg of at least 5 antigens distinguishable by anaphylactic methods, which corresponded to 5 different proteins distinguishable by chemical criteria, clearly demonstrating chemical specificity independent of species specificity. The applicability of these findings to this problem is evident. If we can get from a tissue a protein containing none of the general proteins of the serum, we may expect to get specific reactions for this tissue, but not otherwise.

Abderhalden and Kashiwado,[5] working with nucleoprotein of calves' thymus, by anaphylactic methods, reported a specific reaction. This conclusion was based on their inability to get a reaction when they gave an intoxicating dose of nucleoprotein from duck corpuscles. They conclude that apparently each kind of nucleus produces specific antibodies. Their protocols, however, fail to show whether or not their products were tested against serum of the animals furnishing them in order to exclude general reactions. The chief value of this work was in showing the non-antigenic character of the nucleic acids.

The production of immune serum with the bacterial nucleoproteins has received so much attention that it should be at least mentioned. Lustig[6] gives an excellent review of the literature as well as a considerable contribution to the subject. He

[1] *Jour. Exper. Med.*, 1911, 14, p. 44.

[2] Kolle and Wassermann, *Handbuch d. pathog. Microorg.*, 1913, 2, p. 947.

[3] *Jour. Infect. Dis.*, 1913, 12, p. 341. [4] *Ibid.*, 1911, 9, p. 147.

[5] *Ztschr. f. physiol. Chem.*, 1912, 81, p. 285.

[6] Kolle and Wassermann, *Handbuch d. pathog. Mikroorg.*, 1913, 2, p. 136.

concludes that nucleoproteins are important and chemically definite constituents of bacterial cells and bearers of antigenic functions.

This work with antisera prepared for bacteria seems to come the nearest to showing specificity of the nucleoproteins, but it must be remembered that here we are dealing with single cells in which the chief protein is believed to be a nucleoprotein, and therefore it is doubtful whether it has any advantages as to specificity over the whole cell. Levene's work[1] shows this very clearly. Furthermore, no record can be found of the testing of the antisera against nucleoproteins of bacteria with other proteins of the same bacteria. As a matter of fact the methods employed in the preparation of these so-called nucleoproteins of bacteria are so crude that it seems probable that the substances used under this name are really mixtures of many sorts of proteins.

Inasmuch as the antigenic value of the nucleoproteins has been so much emphasized it seems in keeping to give a brief discussion of these bodies and why they have been so extensively used, especially in the attempted production of antibodies to the complex cells of parenchymatous organs. Beebe appears to believe that some portion of these cells represents their specific properties. He suggests that as the nucleus is the controlling portion of the cell, it must contain this substance. It has commonly been held that the nucleus contains a high percentage of nucleoprotein, hence Beebe thinks that an antibody to this protein will be of highly specific character. This seems reasonable, and at once raises the question: What is a nucleoprotein? This is a difficult question to answer. Hammarsten says that "by nucleoproteins we designate those compound proteins which on cleavage yield protein and nucleic acid, occurring chiefly in nuclei, but widely distributed throughout the body in small amounts." Kossel considers them as proteins combined with a protesteric group, which contains phosphorus, and may be split off as nucleic acid on treatment with an alkali. The protein is a protamine and in some cases a histone, the latter being midway between the former and a protein. It is questioned whether the protamines are true proteins. Kossel considers them the simplest proteins, or as a nucleus of protein bodies. If this be true, then, it would seem that the antigenic properties would be small, for Wells[2] has shown that histone prepared from ripe cod testes possessed no antigenic properties, and Gay and Robertson[3] report that globin, which is a histone, after

[1] *Jour. Med. Research*, 1904, 12, p. 191.

[2] *Jour. Infect. Dis.*, 1911, 9, p. 147.

[3] *Jour. Exper. Med.*, 1913, 17, p. 535.

repeated injection into rabbits, failed to produce an antiserum as shown by the Bordet-Gengou fixation test. The other part, viz., the nucleic acid, which Hammarsten believes gives to the different nucleoproteins their characteristic properties, has been shown by Wells[1] and by Abderhalden and Kashiwado[2] to possess no antigenic properties as shown by the anaphylactic method. As opposed to this, it is of interest to note that Beebe states: "The fact that these bodies are so rich in nucleic acid leads one to believe that the production of antibodies may perhaps be caused as much by the acid portion of the compound as by the proteid." That nucleic acid should be an antigen is, indeed, highly improbable, in view of its relatively simple composition, for there is but little evidence that any substance except complete or nearly complete protein molecules have antigenic functions.[3]

Abderhalden[4] gives the composition of various nucleoproteins which have been studied by several authors. These show rather wide variations, particularly in the phosphorus content. This probably means difference in the amount of nucleic acid in combination with the protein, or in other words, the proportion and kind of protein present. There is no definite line between the nucleoproteins and the nucleins. With our present methods definite separation is probably impossible. Abderhalden[5] in discussing the matter says: "There is little wonder that the existence of the nucleoproteins should be repeatedly questioned." Among those who have made such criticisms may be mentioned Osborne and Harris.[6]

It is evident that different observers working with the nucleoproteins have in all probability been working with widely varying materials. In not a few instances the water-soluble extract has been acidulated and the precipitate, without further treatment, called nucleoprotein. In only a few instances has the material been analyzed. Even in the better preparations where the material is purified, we do not know how much it is changed each time some

[1] *Loc. cit.*

[2] *Loc. cit.*

[3] Wells, *Chemical Pathology* (Second Edition), 1914.

[4] *Handbuch d. Biochem. Arbeitsmeth.*, 1910, 2, p. 449.

[5] *Text Book of Physiological Chemistry*, p. 275.

[6] *Ztschr. f. physiol. Chem.*, 1902, 36, p. 132.

of the protein is split off, or that its character is changed. Wells[1] states: "It seems probable that the numerous chemical manipulations, especially the repeated solution in alkali and precipitation with acid, may be responsible for the inefficiency of these preparations of nucleoprotein and histone, for it is known that the action of acids and alkalies rapidly impairs the activity of proteins in respect to anaphylaxis as well as other biological reactions." As an instance of this he found[2] "that egg albumin converted into alkali albuminate, precipitated with acetic, washed and redissolved in alkali, caused in 0.1 gm. doses no symptoms in pigs sensitized to natural egg albumin." Acid albumin was still active, tho to a less degree than the natural albumin.

The difficulties mentioned would hold in working with simple cells. When we consider that with the placenta we have blood cells, serum, etc., intimately mixed with our placenta cells, the situation is the more complex. On the other hand, if an antiserum can be produced that is specific for placenta by immunizing with any one of the proteins that can be isolated from the placenta, it would seem probable that it might have therapeutic value not attainable with other antisera, for the reason that the placenta is a foreign tissue to the individual in which it is growing. Hence our antisera might be expected to be likely to act on this foreign tissue more exclusively, and to have less effect on the normal permanent tissues of the individual, than would an antiserum for the normal permanent body cells or their proteins.

EXPERIMENTS.

Preparation of materials.—Nucleoprotein of beef liver was first prepared, following Beebe's[3] method as closely as possible. The organs were washed free from blood, sliced, washed several times in running water, and ground to a pulp. This was extracted with two volumes of water in the cold, for 24 hrs., with occasional stirring, a little chloroform being added to prevent bacterial action. It was then strained through cheesecloth with slight pressure. The resulting extract was turbid and contained considerable material in suspension. An attempt was made to clear this by centrifugation, but it was impossible to get the "perfectly clear extract" described by Beebe. This may have been due in part to the fact that it was impossible to get a high enough speed with our machine. Acetic acid was then added to frank acid reaction, and the precipitate allowed to settle out over night, in the ice chest. The super-

[1] *Jour. Infect. Dis.*, 1911, 9, p. 147.

[2] *Ibid.*, 1909, 6, p. 513.

[3] *Jour. Exper. Med.*, 1905, 7, p. 733.

natant clear fluid was then pipetted off, and the remainder, containing the precipitate, filtered on a soft cone filter. This required about 5 hrs. The filtrate was fairly clear and showed only a faint turbidity on the addition of more acetic acid. The precipitate was then suspended in 0.85 per cent saline and washed 4 or 5 times by centrifugation. It was next dissolved in weak sodium carbonate solution, but it was impossible to get a perfectly clear solution. It was then reprecipitated with acid and filtered, this requiring a day or more. After the precipitate was removed it was washed 6–8 times with saline, but the wash-water was never quite clear. Each time it seemed that a little of the material was dissolved. Only a relatively small yield was finally obtained. This was dried over sulfuric acid.

Owing to the difficulties of this method, several modifications were made, eliminating the centrifuge to a large extent. Sheep and dog livers were used in order to perfect the method and to obtain other nucleoproteins for control. In washing the materials free from blood, after the first 2 or 3 rapid washings, a small amount of acetic was added in the hope that it would render the nucleoproteins less soluble. Instead of plain water for extraction of the nucleoproteins, N/20 to N/40 sodium carbonate was used, as the nucleoproteins are more soluble in this, and as, according to Hammarsten, they are insoluble in water. As it was impossible to get a clear extract by centrifugation, various methods of filtration were tried. Finally it was found that the best results were obtained by using a long-fiber asbestos on a Büchner suction filter, prepared in much the same way as an ordinary Gooch crucible. This gave a nearly clear filtrate, from which the protein was precipitated with acetic. When perfectly clear extracts were used the amount of precipitate obtained was decidedly less than when less care was taken to get clear filtrates. Usually the precipitate settles well, and in a short time, a few hours at most, the greater part of the fluid can be pipetted off. The remainder was removed by centrifuge and the precipitate redissolved in N/20 to N/40 sodium carbonate solution. It dissolved fairly well. It was filtered as before with considerable difficulty, again reprecipitated, allowed to settle a short time, filtered, washed several times, first with acidulated and finally with distilled water, which always dissolved some of the material. The precipitate was then removed and dried in a vaccum desiccator over sulfuric acid.

After trying out this method several times, fresh human placentas were obtained and treated in the same way. In all, 42 were used (which were furnished us by the Michael Reese Hospital through the kindness of Dr. J. W. Jobling). From 3 to 12 were worked up at a time. Some lots worked up more easily than others. In general, it was a slow, tedious process, and in some cases but a very small quantity of the nucleoprotein was obtained. In all, about 12 gm. of the thoroughly purified material were prepared.

In addition, other fractions of the placenta proteins were prepared as follows: the *globulin fraction*, by half saturation with $(NH_4)_2SO_4$ of the filtrate obtained after the first precipitation with acetic, purifying by redissolving and again half saturating twice, and removing the salt by dialysis. The *albumin fraction* was obtained by completely saturating the filtrate from the globulin with ammonium sulfate, dissolving the precipitate with the least

possible amount of water, resaturating at least twice, and removing the salt by dialysis.

Gelatin was obtained by treating the residue after the first extraction with weak carbonate to neutralize exactly, and boiling for 2 hrs., filtering and evaporating the hot filtrate to a small volume on the water bath. While still hot, 4 volumes of alcohol were added, which precipitated out the gelatin. After standing a few hours this can be easily filtered. The precipitate was dried a few hours, dissolved in hot water on the water bath, and filtered with considerable difficulty on a hot filter, thus removing all other proteins than gelatin. The gelatin was reprecipitated with alcohol as before, filtered out, and dried over sulfuric acid.

TABLE 1.

Material	Total Nitrogen Percentage	Purine Nitrogen Percentage	Phosphorus Percentage	Millons	Adam-Kewicz	Biuret	Xantho-proteic	Molisch
Nucleoprotein (A). Human placenta	13.35	.....	.....	+++	++	−+	++	o
Nucleoprotein (B). Human placenta	.....	.....	.....	+++	o	o	++	Trace
Nucleoprotein. Sheep liver	.....	.....	.....	++	Trace	±	+	Trace
Nucleoprotein (A). Cow's liver	.....	.....	.....	±	o	o	±	±
Nucleoprotein B^1. Cow's liver 1st ppt	11.76	0.24	0.19	++	+	++	++	o
Nucleoprotein B^2. Cow's liver 5th ppt	11.59	0.19	0.31	++	++	+	++	o
Nucleoprotein. Dog liver	13.56	Trace	0.77	.....	.....	.....	.....	.....
Globulin. Human placenta	15.4	o	o	.....	.....	.....	.....	.....
Albumin. Human placenta	14.56	o	Trace	?	?	o	+++	o
Mucin. Human placenta	.....	.....	.....	+++	+	.....	+++	Trace
Gelatin. Human placenta	.....	.....	.....	+	+	++	+	+

Mucin was prepared from the cords, which had been washed free from blood, finely ground, and extracted with N/10 sodium carbonate for 24 hrs., strained through cloth, and the residue re-extracted 24 hrs. The total extract was then precipitated with N/10 acetic and filtered, washed, and dried in vacuum over sulfuric acid. After thorough drying all these preparations were kept in tightly stoppered bottles in a dry place. The most important of them are characterized briefly in Table 1.

The unreliability of the conventional methods used in the preparation of "nucleoproteins" is well shown by the very irregular character of the material thus obtained, as indicated in Table 1.

Compare, for example, Preparations B^1 and B^2 of nucleoprotein of cow liver. The first represents the first precipitate obtained from a slightly alkalin extract of cow liver, and the second (B^2) is from the same material on redissolving, filtering, and reprecipitating 5 times. It will be noticed that the proportion of purine has been reduced, and the phosphorus increased, during the "purification." Also note that a carefully purified preparation from dog liver yielded only a trace of purine nitrogen, altho made by the usual routine. The phosphorus is very low in all.

Having obtained our materials (the difficulties of this procedure deserve considerable emphasis), and having studied to some extent their chemical composition and reactions, their biological properties were investigated as follows:

ANAPHYLAXIS.

The materials were tested by this method as it readily gives an indication of antigenic properties and specificity. The results of these experiments, given in Table 2, show definitely that the nucleoprotein fraction of placenta is not a good sensitizer, and that no definite symptoms could be elicited on injecting a second dose of the same material after about 20 days' incubation, altho symptoms of varying degrees of severity were obtained on the injection of human serum or the globulin and albumin fractions of placenta into pigs sensitized with nucleoprotein. Furthermore, pigs sensitized to nucleoprotein and failing to react with the same, reacted to human serum the following day. This is in accord with the work of Pearce, Karsner, and Eisenbrey, and of Wells, to which reference has been made. It is noted that positive results were obtained by this method with the globulin and albumin fractions, but that the intoxications were, if anything, more severe when the second dose was human serum rather than the purified protein. In brief, the manifestations were those of a "general human reaction" with no evidence of specificity of the proteins themselves. The nucleoprotein fraction was inert both in sensitizing and in intoxicating properties, particularly the latter. This is not surprising, in view of the fact that the protein part of the nucleoprotein, providing we have such a body, is probably a histone or a protamine,

TABLE 2.

ANAPHYLAXIS EXPERIMENTS WITH PROTEINS.

	Sensitizing Dose	Amount	Second Injection	Amount	Reaction
	NUCLEOPROTEINS FROM	gm.		gm.	
1.	Sheep liver	0.025	Sheep liver nucleoproteins	0.1	Negative
2.	" "	0.010	" " "	0.1	"
3.	" "	0.005	" " "	0.1	"
4.	Dog liver	0.025	Dog " "	0.1	"
5.	" "	0.010	" " "	0.1	"
6.	" "	0.005	" " "	0.1	"
7.	Human placenta	0.05	Human placental nucleoprotein	0.1	"
8.	" "	0.01	" " "	0.1	"
9.	" "	0.005	" " "	0.1	"
				c.c.	
10.	" "	0.05	Human serum	1	Slight
11.	" "	0.01	" "	1	Moderate
12.	" "	0.005	" "	1	Severe
				gm.	
13.	" "	0.001	Human placental nucleoprotein	0.05	Negative*
14.	" "	"	" " "	"	" *
15.	" "	"	Human placental globulin	"	Slight
16.	" "	"	" " "	"	Doubtful
17.	" "	"	Human placental albumin	"	Slight
18.	" "	"	" " "	"	Moderate
19.	Cow liver B^1	0.005	Cow liver nucleoprotein	0.07	Slight
20.	" "	"	" " "	0.07	Slight
21.	" "	"	" " "	0.05	Negative
22.	" "	"	" " "	0.05	"
				c.c.	
23.	" "	"	Beef serum	1	Slight
24.	" "	"	" "	"	Negative
				gm.	
25.	Cow liver B^2	"	Cow liver nucleoprotein B^1	0.07	Slight
26.	" "	"	" " "	"	Severe
27.	" "	"	" " " B^2	0.05	Negative
28.	" "	"	" " " "	"	"
				c.c.	
29.	" "	"	Beef serum	1	Slight
30.	" "	"	" "	"	"
		c.c.		gm.	
31.	Beef serum	0.01	Cow liver nucleoprotein B^1	0.07	Slight†
32.	" "	"	" " "	"	" †
33.	" "	"	" " " B^2	0.05	Negative†
34.	" "	"	" " "	"	"
35.	Human serum	0.1	Human placental nucleoprotein	0.1	"
36.	" "	0.01	" " "	0.05	"
37.	" "	"	" " globulin	"	Moderate
38.	" "	"	" " albumin	"	Severe
		gm.			
39.	Human placental globulin	0.001	" " nucleoprotein	"	Doubtful
40.	" " "	"	" " "	"	"
41.	" " "	"	" " globulin	"	Severe
42.	" " "	"	" " "	"	"
43.	" " "	"	" " albumin	"	"
44.	" " "	"	" " "	"	"
45.	" " "	"	" " "	"	"
46.	Human p acental albumin	"	" " nucleoprotein	"	Slight
47.	" " "	"	" " "	"	Moderate
48.	" " "	"	" " globulin	"	Doubtful
49.	" " "	"	" " "	"	Slight
50.	" " "	"	" " albumin	"	Severe
51.	" " "	"	" " "	"	"
52.	Human mucin from cord	"	" mucin, cord	"	Slight
53.	" " " "	"	" " "	"	Moderate
54.	" " " "	"	Pig stomach mucin	"	Negative‡
55.	" " " "	"	" " "	"	" ‡

* Exper. 13–14 later gave definite reaction to human serum.

† Exper. 31–34 later gave some reaction to beef serum.

‡ Exper. 54–55 next day gave definite reaction to human serum.

both of which are known to be poor antigens or non-antigenic, and the nucleic acid has no antigenic properties.

THE PRECIPITIN COMPLEMENT FIXATION AND PASSIVE ANAPHYLACTIC REACTIONS.

Preparation of the immune sera.—Three pairs of rabbits were injected with nucleoprotein, albumin, and human serum respectively. To the first pair, a total of 2.8 gm. was given in 7 injections intraperitoneally at intervals of 3–4 days. The animals showed no symptoms of toxicity at any time. To the second, a total of 4 gm. was given in 8 doses at the same intervals, by the intravenous method. The first pair treated in this way died on receiving the third dose, with symptoms of acute anaphylactic shock. No evidence of infection could be found on autopsy. Another pair was injected intraperitoneally with no apparent bad results to the animals. The third pair received from 30 to 40 c.c. of human serum in 5 doses by both methods. In all cases the animals were bled to death from the carotid, under ether anesthesia, about 10 days after the last injection; the blood was allowed to clot and the serum was pipetted off and hermetically sealed in tubes. Part was then inactivated, and part kept unheated, all being kept in the refrigerator. The antihuman serum retained its titre almost unimpaired, as shown by both the complement fixation and precipitin tests, for more than a year. The other antisera all kept perfectly also. In the case of one tube of antiserum, however, which contained some red cells it was found that hemolysis was completely or nearly inhibited, which shows the importance of controls for every dilution. The same phenomenon has been noted by Giampalmo.[1] This shows also the importance of having the immune serum perfectly free from red cells, especially when it is allowed to stand so that autolysis may occur.

In the preparation of the hemolytic serum, another interesting point was noted. At the time one of the rabbits was killed she was found to be in advanced pregnancy. After bleeding the mother thoroughly from the carotid, one of the fetuses was carefully removed, its serum obtained and tested with that of the mother.

[1] *Arch. Ital. de Biol.*, 1911, 56, p. 182.

The serum of the fetus gave complete hemolysis in 1:1,000; that of the mother in 1:2,000. The passing over of antibodies through the placenta has long been known, but the amount is seldom stated.

PROPERTIES OF THE VARIOUS ANTISERA.

1. *Precipitin test.*—Precipitins were found in all the antisera prepared, but the antiserum for nucleoprotein was much weaker than the others, that is, only a slight precipitate with a nucleoprotein in a dilution of 1–10,000, and its specific antiserum, while a considerably heavier precipitate was obtained with the same antiserum and human serum diluted 1–40,000, or with the albumin fraction of human placenta. Antiserum to placenta albumin gave quite the same precipitin reactions as the antiserum for nucleoprotein.

Beebe's work[1] with the nucleoproteins on other organs, on the contrary, appears to show specificity. It will be noted, however, that in the protocols given by Beebe the reaction of the immune serum to the serum of the same animal is not shown, nor are the dilutions, etc., given in which the reactions occurred. Pearce[2] was unable to show specificity with any of these fractions by the precipitin reaction.

It is important to run a large number of controls in all this work. Every tube with a different strength of antigen must be run in duplicate against normal rabbit serum. Table 1 shows only the results. Table 3 gives one titration complete as a sample.

Normal serum and antiserum were always used in 0.1 c.c., the several antigens in variable amounts. Readings were taken after 2 hours' incubation and again after standing in the icebox.

2. *Complement fixation.*—Also by this reaction there is no evidence of the specificity of placental nucleoprotein immune serum, for while it reacts with nucleoprotein in 1:100,000 dilution, it also reacts with the globulin fraction and the albumin fraction in just as high dilution, and with human serum in 1:1,000,000 dilution. The antisera for other fractions of placenta proteins also fail to show any specificity, reacting well with high dilutions of human serum;

[1] *Jour. Am. Med. Assn.*, 1910, 55, p. 1714. [2] *Loc. cit.*

and the antiserum for globulin reacts with the albumin as well as with itself, the antialbumin serum and globulin reaction being equally delicate.

These results may be compared with the only other recorded experiments with placental nucleoprotein, namely, those of Frank. He obtained no complement fixation reaction with the serum of rabbits immunized to the nucleoprotein. The antiserum which he obtained by immunizing with suspension of placental cells gave a weak general species reaction, without evidence of specificity for the placenta itself; i.e., much the same result as we obtained with antisera for isolated placenta proteins.

TABLE 3.

PRECIPITIN TEST WITH NUCLEOPROTEIN ANTISERUM.

DILUTION OF ANTIGENS	NUCLEOPROTEIN		GLOBULIN		ALBUMIN		HUMAN SERUM	
	Antiserum	Cont.	Antiserum	Cont.	Antiserum	Cont.	Antiserum	Cont.
1:400.....	Turbid	Clear	Sl. turbid	Clear	Heavy ppt.	Heavy	Ppt.	Clear
1:2,000.....	"	"	" ppt.	"	Med. ppt.	Med. ppt.	"	"
1:4,000.....	Ppt.	"	Def. ppt.	"	Light "	Light "	"	"
1:10,000.....	Sl. ppt.	"	" "	"	Trace	Trace	"	"
1:20,000.....	Trace?	"	" "	"	"	"	"	"
1:40,000.....	Clear	"	" "	"	Def. ppt.	Clear	"	"
1:100,000.....	"	"	" "	"	Clear	"	Clear	"
1:200,000.....	"	"	Sl. ppt.	"	Sl. ppt.	"	"	"
1:400,000.....	"	"	Clear	"	Clear	"	"	"
No antigen...	"	"	"	"	"	"	"	"

3. *Passive anaphylaxis test.*—Inasmuch as this test was used in checking up the reactions of immune sera prepared for some of the vegetable proteins (see p. 364), with good results, it seemed well to use it here for comparative purposes. The test was carried out as follows: Guinea-pigs of about 300 gm. are given injections of from 1 to 3 c.c.of the immune serum and 24–72 hrs. later an intoxicating dose of the specific antigen, when, in case of a positive result the typical symptoms of acute anaphylaxis will follow. The guinea-pigs receiving serum from a rabbit immunized with nucleoprotein showed no symptoms whatever when given an injection of the same nucleoprotein. The animals sensitized with antialbumin serum did not react to a toxic dose of nucleoprotein, but did react to both the globulin and albumin fractions of placenta. The chief

results obtained with the antisera described above are summarized in Table 4.

Had the results been more favorable up to this point, it was the intention to try the other tests commonly used as well, i.e., agglutination and lysis of cell suspensions, and injections of specific antisera into animals. From the work of all the other observers, however, apparently no data of importance have been gained by the agglutination tests that are not also obtained with the reactions

TABLE 4.

GENERAL REACTIONS OF THE ANTISERA.*

	ANTIGENS	ANTISERA				
		Antiserum for Egg White	Antiserum for Human Serum	Antiserum for Placental Nucleo-protein	Antiserum for Placental Albumin	
					(A)	(B)
Precipitin reaction	Egg white	1:200,000				
	Human serum		1:100,000	1:40,000	1:100,000	1:400,000
	Placental nucleoprotein		1:100 (?)	1:10,000	Negative	Negative
	" globulin		1:100,000	1:100,000	1:10,000	1:8,000
	" albumin		1:10,000	1:200,000	1:40,000	1:80,000
	Mucin from cord		1:100			
Complement fixation	Egg white	1:1,000,000				
	Human serum		1:1,000,000	1:1,000,000	1:1,000,000	1:500,000
	Placental nucleoprotein		1:10,000	1:100,000	1:50,000	1:100,000
	" globulin		1:100,000	1:100,000	1:50,000	1:250,000
	" albumin		1:100,000	1:100,000	1:500,000	1:100,000
	Mucin from cord		1:10,000			
Passive Anaphylaxis	Egg white	Severe				
	Placental nucleoprotein			Negative	Negative	
	" globulin				Moderate	
	" albumin				Moderate	

* The figures indicate the highest dilutions, in the case of the precipitin reactions, of the antigens, and in case of complement fixation, of the antisera in which the reactions are positive.

that we have used. Further, it hardly seems that the agglutination tests can be as delicate as the complement fixation tests. In the case of cholera and typhoid, where the agglutination tests are largely used, it is more because of the ease with which they are carried out. As a matter of fact, the other tests would probably be far more delicate. With the placenta it would be hard to get a uniform cell suspension, and besides it would seem that the results are about as definite without this.

In regard to testing the specificity of the antiserum by injecting it into animals and seeking for specific lesions, it would seem rather

crude to attempt this with a serum for which it is impossible to show specificity by such delicate methods as those already used.

CONCLUSIONS.

It is believed, therefore, that the following conclusions are warranted:

1. The nature of nucleoproteins in general is not well known; in fact, they may not exist at all as definite and constant chemical compounds, and it is very doubtful if the "nucleoproteins" isolated from tissue extracts correspond to compounds actually present in the living cells.

2. The "nucleoproteins" studied in these experiments, and in the case of the placenta, the globulin, albumin, and gelatin fractions as well, fail to show organ specificity. The reactions obtained may all be accounted for on the basis of general species reaction. Proteins showing independent specificity could not be isolated from placenta.

3. Antisera prepared with the several protein fractions of the human placenta react as well with human serum as with the isolated placenta proteins themselves; and they showed no definite specificity between themselves.

4. From this we conclude that the possibility that immune serum of therapeutic value in chorion-epithelioma can be prepared specifically for the human placenta is, at least at this time, extremely slight.